DESCRIPTION

GENE PANEL PARTICIPATIVE IN HEPATIC STELLATE CELL ACTIVATION

Technical Field

The present invention relates to a gene panel comprising genes each showing an increased expression level in hepatic stellate cells in accordance with activation of the hepatic stellate cells compared with that in a normal state, a method for producing therefor, and a use of the gene panel. The present invention is useful in the field of diagnosis, pharmaceutical, or the like.

Background Art

Hyperplasia and accumulation of hepatic connective tissues lead to circulatory disturbance of the liver. It is considered that this also causes hepatocyte injury, thereby forming a vicious cycle in which further hyperplasia and accumulation occur, resulting in liver disease accompanying liver cirrhosis and hepatic fibrosis in a hepatic fibrosis model animal. The liver consists of parenchymal cells (hepatocyte) and non-non-parenchymal cells (hepatic stellate cells, Kupffer cells, sinusoidal endothelial cells, and Pit cells), and the hepatic connective tissue is constituted of extracellular matrix and cells localized therein. Activation and transformation of hepatic stellate cells, which are stroma producing cells in connective tissues, promote hyperplasia and accumulation of the connective tissues. It is known that the hepatic stellate cell of

normal liver (hereinafter referred to as (quiescent) stellate cell in a resting state) produces the extracellular matrix in a small amount, is transformed into a myofibroblast-like cell in accordance with the activation and synthesizes a large amount of extracellular matrix together with the increase of the cells.

Therefore, there is required a medical care in which the activation of the hepatic stellate cells in a patient suffering from liver disease with hepatic fibrosis such as cirrhosis is suppressed to thereby alleviate hyperplasia and accumulation of the connective tissue. Thus, it can be said that screening for drugs which inhibit the activation of the hepatic stellate cells is important. Despite the above requirement, there has not been known an effective method of screening drugs inhibiting the activation of hepatic stellate cells.

Once hepatic cells have come to necrosis by hepatic disease, the hepatic stellate cells which have been in a resting state till then are activated by humoral factors derived from the hepatic necrosis cells, cytokines being paracrine-secreted from the activated Kupffer cells at inflammatory local sites or invaded inflammatory cells, and further autocrine-secretion by the activated hepatic stellate cells. Note that, a plurality of factors are considered to cause the activation of the hepatic stellate cells, but it is generally uncertain what timing they act at. It is thus important to study a plurality of essential genes acting for the hepatic stellate cell activation for the purpose of grasping the whole image of the hepatic stellate cell

activation, because a plurality of genes are considered to cause the hepatic stellate cell activation. No drugs have ever been produced from screening of the drugs suppressing hepatic stellate cell activation. There has been reported gene screening in which the observation of expression changes during hepatic stellate cell activation is used as index. However, in the above gene screening, the observation of expression changes is carried out on at most several kinds of genes.

Disclosure of the Invention

The present invention has been made in view of the above and it is an object of the invention to provide a gene panel comprising genes showing a change in an expression level in a state of hepatic stellate cell activation, compared with a resting state of hepatic stellate cells, and a method of screening a drug that inhibits the activation of hepatic stellate cells.

The present inventors considered that, for inhibiting the activation of hepatic stellate cells, a behavior of genes related to the activation of hepatic stellate cells should be made similar to a gene expression pattern of hepatic stellate cells in the resting state. In addition, the invention has been completed as a result of investigating expression profiles of various genes in the activation of hepatic stellate cells and obtaining information about the expression of genes related to the hepatic stellate cells using an activation model in a primary culture system of hepatic stellate cells.

More specifically, the present invention is as follows.

(1) A gene panel comprising names and gene expression profiles of genes each showing, in hepatic stellate cells, an increased expression level in an activation state compared with a level in a resting state.

(2) The gene panel as described in the item (1) or (2), in which the increased expression level of the gene corresponds to a difference of an expression level in a model animal having liver cirrhosis and hepatic fibrosis with an expression level in a normal state in a model animal.

(3) The gene panel as described in the item (1) or (2), in which the expression profile comprises a time-varying expression profile in the hepatic stellate cells.

(4) The gene panel as described in the item (2) or (3), in which the model animal is a rat.

(5) The gene panel as described in any one of the items (1) to (4), further comprising an expression profile of each of at least 5 kinds of genes among 105 kinds of genes represented as Nos. 1 to 105 listed in Tables 1 to 4.

(6) A method of producing a gene panel comprising genes each showing, in hepatic stellate cells, a varied expression level in a hepatic stellate cell activation state compared with a level in a normal state, comprising the steps of:

(a) measuring expression levels of various genes in the hepatic stellate cells in the resting state and the expression levels of the genes in the hepatic stellate cells in the activation state;

(b) comparing the expression levels with each other; and

(c) identifying the genes showing the increased expression level in the activation state.

(7) The method as described in the item (6), wherein the expression levels of the genes in the hepatic stellate cells activation state are analyzed in time course in the step (a).

(8) The method as described in the item (6) or (7), wherein the expression levels of the gene are analyzed by a gene chip method.

(9) A method for screening a drug related to hepatic stellate cell activation, comprising the steps of administering the drug to a model animal or hepatic tissues or cells, and profiling expressions of genes constructing the gene panel as described in the item (1).

Hereinafter, the present invention will be described in detail.

<1> Gene panel of the present invention

The gene panel of the present invention is a gene panel comprising the names of the genes, each of which shows an increase in its expression level in hepatic stellate cells in an activation state , compared with the resting state thereof; and their respective gene expression profiles.

The gene panel of the present invention can be produced in the steps of:

(a) measuring expression levels of various genes in a hepatic stellate cell in a resting state and expression levels of the genes in hepatic stellate cells in an activation state;

(b) comparing the expression levels with each other; and

(c) identifying genes showing an increased

expression level in the activation state.

The functions of hepatic stellate cells include the metabolism and storage of vitamin A, the production of extracellular matrix, the production of cytokines, contractility, and the transformation to myofibroblast cells. These functions are considered to be closely related to hepatic fibrosis. The term "hepatic stellate cells in an activation state (synonymous with activated hepatic stellate cells or active-type hepatic stellate cells)" means hepatic stellate cells in a state of being transformed from hepatic stellate cells in the resting state of the normal liver to myofibroblasts under various stimuli. The cell transformed to the myofibroblast shows an increase in proliferative property, a decrease in the content of vitamin A, and an increase in contractility. In addition, the cell produces an extracellular matrix that mainly contains type I collagen. Furthermore, the synthesis of an extracellular matrix catabolic enzyme such as collagenase or gelatinase slows down, so that the extracellular matrix can be increased and accumulated.

The activated hepatic stellate cells have been known for causing similar transformation in isolated hepatic stellate cells. In hepatic diseases such as hepatitis (J hepatology 1996 Mar 24, (3):301-7 Guido M liver stellate cells in chronic viral hepatitis: the effect of interferon therapy), cirrhosis (American Journal of pathology 1990 July 137(1) Stefano M et al. Cellular Localization of Type I, III, and IV Procollagen Gene transcripts in Normal and Fibrotic Human liver), and NASH (nonalcoholic steatohepatitis)(Hum Pathol 2000 July 31(7)822-8, Washington K, et al Hepatic stellate

cell activation in nonalcoholicsteatohepatitis and fatty liver.), an increase in number of the active-type hepatic stellate cells is observed as connective tissues proliferate and accumulate in the liver with its sustained inflammatory.

The term "expression level of a gene" is synonymous with the expression amount, expression intensity, or expression frequency of a gene, which is analyzed with the production amount of a translation product corresponding to the gene, the activity of the translation product, or the like.

The expression level of the gene can be measured using any method generally used for the analysis of gene expression. Preferable methods include a gene chip method, a gene microarray method, and a gene macroarray method. Each of them arranges and attaches gene fragments on any plate (typically, slide glass). It allows the chip to hybridize with fluorescence-labeled mRNA to quantify and specify the mRNA.

Alternatively, other methods of analyzing the gene expression include an ATAC-PCR method (Nucleic Acids Research 25,4694-4696(1997)), a Body Map method (Gene, 174, 151-158(1996)), the serial analysis of gene expression (SAGE) (US527,154B, US544,861B and EP0761,822A), and a MAGE (Micro-analysis of Gene Expression) (JP 2000-232888A).

The ATAC-PCR method will be outlined as follows. At first, a double-stranded DNA is prepared from cDNA synthesized with a 5'-biotinylated oligo-dT primer and is then digested with a given restriction enzyme (the example using *Mbo*I will be described herein). Subsequently, adaptors having a sequence common to the

portions cleaved with the restriction enzyme (preparing six adaptors having different lengths) and the double-stranded DNA cut out with the restriction enzyme *Mbo*I are ligated with each other by a DNA ligase. Furthermore, among these six different adaptors, three of them are coupled to the respective control cDNAs (cDNAs prepared from hepatic stellate cells in the activation state) and then mixed at a ratio of 10:3:1. The remaining three adaptors are coupled to cDNAs prepared from rat hepatic stellate cells cultured for 4 hours, 3 days, and 7 days in non-coating plastic petri-dishes, respectively.

After mixing the respective ligation products, the 3'-fragment of the double-stranded cDNA is recovered using streptavidin-coated beads to perform a competitive RT-PCR by using primers having sequences common to the respective adaptors. The PCR product is analyzed with the ABI PRISM 3700 DNA Analyzer. This system is able to isolate fragments for each of different lengths thereof by capillary electrophoresis, so that the intensity of fluorescence can be detected in proportion to the expression amount.

The Body Map method will be outlined as follows: cDNA is prepared from mRNA using the poly-T sequence of a vector as a primer such that the poly-A tail of the 3' end of the mRNA is coupled to the vector's poly-T sequence. Furthermore, the cDNA is cut out with the restriction enzyme *Mbo*I. As the cDNA has an *MBo*I site every 300 base pairs on average, the cDNA on the vector will be cleaved every 300 base pairs on average. At this time, the cDNA proximate to the poly-A tail still remains coupled to the vector. The vector having this

cDNA fragment is cyclized and then introduced in *E. coli* to prepare a cDNA library. About 1,000 clones are arbitrary selected from the library and the nucleotide sequences having 300 base pairs on average are defined for the respective clones. Among these sequences, clones are sorted out into groups such that each group includes clones having the same sequence. Then, these sequence kinds and the expression frequencies of the respective sequences are investigated and calculated to obtain a gene expression profile. Each cDNA sequence is subjected to a search for the homology thereof to a data bank (the BLAST search). When the clone has the same sequence as that of a known gene, the name of the gene is given to the clone. When the sequence has not been registered in the data bank, it is assumed that any gene corresponding to such a sequence is not present.

For conducting the homology search with the BLAST search, the information of at least 11 base pairs will be required. There are about one million kinds of a sequence consisting of 10 bases, exceeding the number of gene types (100,000 types) expected to be present in human beings. In other words, the information about 11 base pairs makes it possible to specify the gene having the sequence, allowing a gene expression profile analysis. Therefore, for more efficiently conducting the gene expression profile analysis with the Body Map that requires a great number of sequences, cDNA fragments of about 300 base pairs in the Body Map are made into shorter fragments of 11 or more base pairs (referred to as "tags"). Then, these fragments are linked together in large quantity and then inserted into a vector. Consequently, a linked tag library is

prepared and then about 1,000 clones are arbitrary selected in the same manner as that of the Body Map. Determining the DNA sequence of the linked tag can be expected to obtain more information about gene expression in the same procedure as that of the Body Map. The tag represents a gene sequence and the appearance frequency of the tag corresponds to the expression frequency of the gene thereof. Generally, the length of a DNA sequence which can be read out per sequencing is about 600 base pairs, so that the DNA sequences of about 50 tag DNAs can be read out at the maximum through one sequencing. In other words, the gene expression profile analysis can be performed with an efficiency about 50 times as high as that of the Body Map method.

The SAGE method is a gene expression profile analysis based on the above ideas. The SAGE method will be conducted as follows: cDNA is prepared using poly-T where the 3'-end thereof is coupled to biotin is used as a primer. Similarly to the Body Map, cDNA as a primer is prepared using poly-T having the 3'-end coupled to biotin and then the cDNA is cut out by a restriction enzyme such as *Mbo*I (referred to as an "anchoring enzyme"). After that, cDNA fragments containing the biotin-coupled 3'-ends are adsorbed on avidin beads, followed by dividing the beads into two groups. Then, one of two linkers (A or B) is coupled to the cDNA fragment (about 13 bp) adsorbed on one of bead groups. In each linker, the site of a Class-II restriction enzyme such as *Bsm*FI (referred to as a "tagging enzyme") is included in advance. The tagging enzyme cuts the cDNA fragment out of the beads and flattens the cleavage site thereof to link the tag coupled to the A linker

with the tag coupled to the B inker. It is referred to as a "ditag". The ditag is amplified by PCR using a primer that recognizes both the A linker and the B linker. The great number of amplified ditags are coupled together and then incorporated into a vector, followed by sequencing. About 50 tag sequences can be read out at the maximum through one sequencing. The frequency of gene expression can be derived from the compiled information about the tag sequence.

The MAGE method is an improved method of the above methods. The method is capable of analyzing the expression frequency of a gene efficiently with high precision by preparing cDNA from mRNA using a vector primer having a poly-T sequence, tagging the cDNA sequence on the vector, ligating the resulting tags through the intermediation of a sequence capable of recognizing the end of the tag to form a concatamer, and analyzing the nucleotide sequence of the concatamer.

In the present invention, the method is not specifically limited as far as it can analyze the expression level of a gene. Any method presently known in the art or method to be developed in future can be adopted. Among the above methods, particularly preferable methods include a gene chip method, a gene microarray method, and an ATAC-PCR method.

In the present invention, the analysis of gene expression may be performed depending on the result obtained by a single method or may be performed by combining the results obtained from two or more methods. Even though the single method allows the analysis, the combination of two or more methods allows a more precise analysis. Specifically, the coefficient of correlation

between the results of two or more methods, for example, between a result obtained by the gene chip method and a result obtained by the ATAC-PCR method, is calculated. Then, the gene having a correlation coefficient above a certain level is evaluated as being changed in the expression level.

Various gene chips of humans and animals such as mice and also various gene micro- and macro-arrays are commercially available, so that the present invention may adopt one of them.

In the present invention, a change in expression level of a gene can be analyzed by measuring the expression levels of various genes in hepatic stellate cells in the resting state and the expression levels of these genes in the hepatic stellate cells in the activation state and making a comparison between the respective expression levels.

The expression level of the gene in the hepatic stellate cells in the resting state can be analyzed by measuring the expression level of the gene in the hepatic stellate cells directly after isolating from a normal liver.

On the other hand, the expression level of the gene in the hepatic stellate cells in the activation state can be analyzed by, for example, incubating hepatic stellate cells isolated from a normal liver as a primary culture in a non-coating plastic petri-dish, and measuring the expression level of the gene in the resulting cells. Only by incubating the hepatic stellate cells isolated from the normal liver as a primary culture in the non-coating plastic petri-dish, the cells activate themselves to show traits similar to

those of the active-type hepatic stellate cells observed in the liver tissues of a clinical sample such as cirrhosis or hepatitis, for example, the decrease in accumulation of lipid droplets, and the production of extracellular matrix.

When the hepatic stellate cells are isolated from the normal liver of a rat and inoculated in a non-coated plastic petri-dish, many lipid droplets for storing vitamin A are observed in the cytoplasm of the hepatic stellate cells directly after the isolation, showing the form similar to the hepatic stellate cells of the normal liver in the resting state. After isolating the hepatic stellate cells and incubating them on a non-coating plastic petri-dish, α-smooth muscle actin, which is a marker of the activation of hepatic stellate cells, is observed 3 days after the culture, and after about 1 week, the hepatic stellate cells show the myofibroblast-like form as activated hepatic stellate cells. The activated hepatic stellate cells on the seventh day shows the form similar to that of the activated hepatic stellate cells in which lipid droplets to be observed in cirrhosis, fibrosing liver, or the like are diminished.

In addition, the expression level of a gene in hepatic stellate cells in the activation state is analyzed by measuring, for example, the expression level of the gene in the hepatic stellate cells just after isolating them from the liver of a cirrhosis /liver-fibrosing model animal. On the other hand, the expression level of the gene in the resting state is measured directly after isolating hepatic stellate cells from the liver of a normal model animal. It is preferable to analyze the expression level of the gene

in time course as the hepatic stellate cells are activated.

The cirrhosis/liver-fibrosing model animal can be obtained by, for example, intraperitoneally administrating 1 ml thioacetamide in physiological saline (50 mg/ml) to a male rat twice a week for six weeks. During this period, the rat is bred in free-feeding and free-drinking.

As described above, the genes in which the expression level thereof varies in the activation state, compared with genes in the resting state, are identified.

The gene panel of the present invention includes at least the names of various genes measured as described above and the expression profiles of the respective genes, i.e., information about a change in expression level. A nomenclature for the name of a gene is not specifically limited as far as the name can be distinguished from the names of other genes. Typically, the name of the product encoded by the gene, the accession number or genetic name on a data base such as the GeneBank, the name of a probe set or the name of the gene on a gene chip, or the like is used.

In a preferred embodiment of the gene panel of the present invention, genes are classified depending on the expression level thereof after a given period of time from the isolation of hepatic stellate cells. For instance, the genes are classified into groups of an expression amount increasing after three days (at an initial stage of activation) and that increasing after seven days (the active-type hepatic stellate cells) from the isolation of hepatic stellate cells. The term remarkable used herein means that the expression amount

is increased more than 3-fold as compared with that of the hepatic stellate cells in the resting state.

<2> Screening method of the present invention

On the basis of the gene panel of the present invention, various kinds of screening is made possible by constructing a system for quantitatively or semi-quantitatively measuring the expression of a gene included in the gene panel.

For instance, it is possible to perform screening on a drug related to hepatic stellate cells by administering a drug to a hepatitis, cirrhosis, or NASH model animal or activated hepatic stellate cells and profiling the expressions of the respective genes that constitute the gene panel of the present invention. In other words, it is considered that the administration of the drug may inhibit the activation of hepatic stellate cells if the drug is one where the expression profile of each gene is similar to the expression profile in the hepatic stellate cell gene panel in the resting state.

Furthermore, a substance for inhibiting the activation of hepatic stellate cells can be also screened by screening a drug for further promoting an increase in expression of a gene in this gene panel or a drug for further promoting a reduction in expression thereof. In this case, the screening is made possible by focusing on changes in expressions of almost five kinds of genes.

The methods of profiling the expression of a gene include a DNA microarray method, a DNA macroarray method, or an ATAC-PCR method, a method using a Taqman probe, a quantitative PCR method using SYBR Green, or the like

using slide glass, a nylon membrane, or the like on which fragments of genes that constitute the gene panel are fixed. The expression profiling may be performed by a single method or a combination of two or more methods.

In the following, an example of the specific screening procedures will be described.

As primary screening, isolated activated hepatic stellate cells or established activated hepatic stellate cells are treated with a screening-target drug and then RNA is prepared from the cells after a given period of time. The stellate cells isolated from the liver become active as time goes by, so that the action of the drug may be measured on the cells, which are provided as targets, at each stage of the activation, for example, an initial stage of activation (from the time immediately after the isolation to the third day of the incubation), a middle stage (from the fourth day to the sixth day of the incubation), and a later stage (from the seventh day forward of the incubation). Measuring the expression level of a gene before and after drug treatment, screening is performed for a drug where the expression profile is analogous to the gene panel of stellate cells in the resting state or a drug that inhibits the expression of a gene to be increased with the activation thereof. The gene expression patters in hepatic stellate cells after the drug treatment are sorted into groups, followed by screening a drug analogous to the profile of the gene expression panel in the resting state or a drug that inhibits the expression of a gene to be increased with the activation thereof. Grouping is performed through classification based on the expression level for a given period of time

similarly to the preparation of the gene panel.

Proteins provided as gene expression products in the cells are related with each other and form networks. The networks are constructed by direct binding, establishing the relationship between an enzyme and a substrate, and controlling the transcription of a specific gene such that the protein binds to the specific site of the genome, respectively. In the activation of the hepatic stellate cells, a series of networks is actuated and then the actuation thereof actuates the subsequent series of networks. It is conceivable to provide a cascade in which the actuation actuates the subsequent network. As a result of this cascade, the hepatic stellate cells are activated and finally developed into myofibroblasts. This panel is one collectively including changes in expressions of all genes related to such a cascade. For the requirements of a hepatic stellate cell activation inhibitor, it is desired to stop the network by inhibiting the network on the upstream side or stopping the cascade by inhibiting a signal transmission around a transcription factor, via the transcription factor. From the above, the promising inhibitor may generate gene changes in this panel together. This assembly is expected to include about 10 genes related to one network in this panel, although depending on the occasion. Thus, at least five genes are expected to be changed by one inhibitor. Therefore, changes in at least five genes are involved in a judgment on the effects as primary screening.

As the conditions for culturing hepatic stellate cells, there are methods including the incubation in a non-coated plastic petri-dish, the addition of a

substance that accelerates the activation of hepatic stellate cells, such as TGF-1β (tumor growth factor), and so on. It is expected that a substance for inhibiting the activation of hepatic stellate cells or a substance for inhibiting the proliferation of hepatic stellate cells will be screened.

As secondary screening, a candidate drug expected to inhibit the hepatic stellate cell activation or the hepatic stellate cell proliferation in the primary screening is administered to a pathologic model rat prepared using a hepatitis-inducing agent, a cirrhosis-inducing agent, a fatty liver-inducing agent, or the like and then the lever is enucleated from the rat. The amount of connective tissues in the liver is measured, while the RNA thereof is extracted to measure a change in gene expression. The data thereof is compared with the data of a change in gene expression which is measured while measuring an amount of the liver connective tissues of the rat administered with no drug to evaluate the effects of the drug on the proliferation or accumulation of liver connective tissues.

It is possible to carry out the above screening using an experimental animal other than a rat. In this case, it is preferable to perform the screening by rebuilding a gene panel with respect to a homolog corresponding to a rat gene.

As described above, the gene panel of the present invention is considered to be useful in screening of an effective drug for the activation of hepatic stellate cells. As a result, in combination with the resulting drugs, supposedly, it is also possible to create a more effective remedy for the hepatic fibrosis.

Brief Description of the Drawing

Fig. 1 shows an amount of hydroxyproline (Hyp) in the liver of a rat fed with casein or L-cysteine.

Best Mode for carrying out the Invention

Hereinafter, the present invention will be described in more detail based on examples.

Example 1

<1> Isolation of hepatic stellate cells

A male Wistar rat (300-350 g in body weight) was anesthetized with ether, 0.5 ml of Nembutal was then intraperitoneally injected, and a portal vein is exposed by performing laparotomy in Cooper. Then, Surflo is inserted into the portal vein slightly from the periphery side.

200 ml of a perfusate (8 g NaCl, 400 mg KCl, 88.17 mg $NaH_2PO_4 \cdot 2H_2O$, 120.45 mg Na_2HPO_4, 2380 mg HEPES, 350 mg $NaHCO_3$, and 560 mg $CaCl_2 \cdot H_2O$ per litter), 100 ml of a 70-mg pronase perfusate, and 250 ml of a 70 mg collagenase perfusate were circulated. After perfusion, the liver was extracted and then the cells thereof were dispersed. These cells were further digested in a perfusate containing 70 mg pronase, 70 mg collagenase, and 2 mg DNaseI.

After that, the cell dispersion liquid was filtrated through a mesh device and the resulting filtrate was then dispensed into two Falcon tubes, followed by centrifugation at 2000 rpm for 7 minutes. A supernatant was discarded and 0.5 mg DNaseI was then added, and furthermore an agitating solution (8 g NaCl, 370 mg KCl, 210 mg $MgCl_2 \cdot 6H_2O$, 70 mg $MgSO_4 \cdot 7H_2O$, 150 mg

$Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, 991 mg glucose, 227 mg $NaHCO_3$, and 225 mg $CaCl_2$ per litter) was added and pipetted, followed by centrifugation at 2000 rpm for 7 minutes. A supernatant was discarded and then 67.5 ml of the agitating solution was added to suspend the cells. Then, a Nicodenz solution (7.75 g Nicodenz was dissolved in 25 ml of solution containing 370 mg KCl, 210 mg $MgCl_2 \cdot 6H_2O$, 70 mg $MgSO_4 \cdot 7H_2O$, 150 mg $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, 991 mg glucose, 227 mg $NaHCO_3$, and 225 mg $CaCl_2$ per litter) was added and stirred, and then dispersed into centrifuge tubes. In each of the tubes, 1 ml of the agitating solution was layered. Then, it was centrifuged at 3200 rpm for 15 minutes.

A cell layer was obtained at the lower face of the top layer, and then this layer was sucked and transferred to the centrifuge tube. The agitating solution was added to suspend the cells, followed by centrifugation at 2000 rpm for 7 minutes. A precipitate was suspended in 10%FCS-added DMEM and then seeded on a non-coating plastic petri-dish. After 4 hours, a culture solution was replaced with 10%FCS-added DMEM and also cells attached on the petri-dish were defined as hepatic stellate cells in the resting state. Furthermore, the cells attached on the petri-dish after cultured for 3 days and 7 days were provided as hepatic stellate cells at an initial stage of the activation and the activated hepatic stellate cells, respectively.

<2> Purification of total RNA

For 10^7 cells, 1 ml of ISOGEN (Nippon Gene Co., Ltd.) was added and homogenized. The obtained homogenate was centrifuged and a supernatant was then

recovered. In this supernatant, 200 μl of chloroform was added per milliliter of ISOGEN and gently stirred. After being left to stand for 2 minutes at room temperature, the resultant was centrifuged at 15000 rpm at 4° C for 10 minutes. Then, an aqueous layer was transferred to a new centrifuge tube. Then, an equal amount of 2-propanol was added to the aqueous layer and then left standing at room temperature for 5 minutes, followed by centrifugation at 15000 rpm at 4° C for 15 minutes. The supernatant was discarded and 70% ethanol was added to precipitated pellets, followed by centrifugation at 15000 rpm at 4° C for 15 minutes. Subsequently, 70% ethanol was removed while the pellet was rinsed. Then, the rinsed pellet was dried at room temperature for 5 minutes and DEPC (diethyl pyrocarbonate)-treated water was added to dissolve the pellet. Using 1% agarose gel electrophoresis, it was confirmed that the total RNA fraction thus obtained was purified.

As described above, the total RNA was purified from each of hepatic stellate cells after isolation, after 3 days of incubation, and after 7 days of incubation and then the change of the gene expression amounts were investigated using GeneChip (manufactured by Affymetrix Co., Ltd.).

<3> Gene expression analysis with GeneChip

The gene expression analysis with GeneChip was carried out in accordance with the protocol recommended by Affymetrix Co., Ltd. The procedures are as follows:

(1) Probe synthesis

(i) Double-strand cDNA synthesis

At first, from the total RNA prepared in <2>, a double-stranded cDNA was synthesized using the SUPERSCRIPT Choice System manufactured by Gibco BRL Co., Ltd. 15 μg of the total RNA and 100 pmol of T7-$(dT)_{24}$ primer were dissolved in a DEPC-treated water so as to be 11 μl in volume. After reacting at 70° C for 10 minutes, it was cooled with ice and then 4 μl of a 5 x 1st strand cDNA buffer (manufactured by Gibco BRL, Co., Ltd.), 2 μl of a 0.1 x DTT (dithiothreitol, manufactured by Gibco BRL, Co., Ltd.), and 1 μl of a 10-mM dNTP mix (manufactured by Gibco BRL, Co., Ltd.) were added and kept at 42° C for 2 minutes. Then, 2 μg of a reverse transcriptase (Superscript II RT) was added therein, followed by reacting at 42° C for 1 hour.

In the reaction solution, the DEPC processing solution, 30 μl of 5 x 2nd strand reaction buffer, 3 μl of 10-mM dNTP, 1 μl of DNA ligase (10 U/μl), 4 μl of DNA polymerase I (10 U/μl), and RNaseH (2 U/μl) were added and mixed, followed by reacting at 16° C for 2 hours. Subsequently, 2 μl of T4 DNA polymerase (5U/μl) was added and reacted at 16° C for 5 minutes. Then, 10 μl of 0.5M EDTA was added therein. In the reaction solution, an equal amount of a (phenol:chloroform = 1:1) solution was added. Then, a tube containing these components was shaken up and down to mix them together. The mixture solution was then subjected to the centrifugation at 15000 rpm at 4° C for 10 minutes. Then, an aqueous layer was transferred into a new centrifuge tube. A 1/10-fold volume of 3M sodium acetate and a 3-fold volume of 100% ethanol were added to the aqueous layer and mixed well. It was left standing at -80° C for 10 minutes, followed by centrifugation at 15000 rpm at 4° C for 10 minutes.

The precipitated pellet was rinsed twice with 70% ethanol and dried at room temperature for 5 minutes, followed by adding 12 μl of the DEPC processing solution.

(ii) Synthesis of biotin-labeled cRNA probe

Next, from the double-stranded cDNA thus synthesized, a biotin-labeled cRNA probe was synthesized using the Bio Array High Yield RNA Transcript Labeling Kit, manufactured by Enzo Co., Ltd. Then, 5 μl of the double-stranded cDNA, 17 μl of the DEPC processing solution, 4 μl of a 10 x HY buffer, 4 μl of a 10 x Biotin labeled ribonucleotides, 4 μl of 10 x DTT, 4 μl of a 10 x RNase inhibitor mix, and 2 μl of 20 x T7 RNA polymerase were mixed together and reacted at 37° C for 4 hours.

Next, from the biotin-labeled cRNA probe solution synthesized as described above, unreacted Biotin labeled ribonucleotides were removed using RNeasy, manufactured by Qiagen Co., Ltd. In the biotin-labeled cRNA probe solution, 160 μl of the DEPC processing solution was added and mixed with 700 μl of RLT buffer, and 500 μl of 100% ethanol was also added and mixed well. A 700-μl aliquot of the solution was added to each of RNeasy mini spin columns and centrifuged at 8000 rpm for 15 seconds. The resulting eluent was added to the RNeasy mini spin column again and centrifuged at 8000 rpm for 15 seconds. Subsequently, 500 μl of RPE buffer was added to the RNeasy mini spin column and then centrifuged at 8000 rpm for 15 seconds. Then, 500 μl of the RPE buffer was added to the RNeasy mini spin column again and centrifuged at 15000 rpm for 2 minutes.

As described above, the RNeasy mini spin column, on which the biotin-labeled cRNA probe was adsorbed, was

washed and transferred into a new centrifuge tube. In the RNeasy mini spin column, 30 μl of the DEPC processing solution was added and left standing at room temperature for 1 minute. It was centrifuged at 8000 rpm for 15 seconds, followed by eluting the purified biotin-labeled cRNA probe solution.

Subsequently, the purified biotin-labeled cRNA probe solution was fragmented. The biotin-labeled cRNA probe solution and a 5 x Fragmentation buffer (1/5-fold volume of the final amount of the solution) were mixed and adjusted such that the concentration of the biotin-labeled cRNA probe becomes 0.5 μg/μl, and then reacted at 94° C for 35 minutes. Performing 1% agarose gel electrophoresis, it was confirmed that the probe can be fragmented into fragments each having a length of around 100 base pairs.

(2) Hybridization

For the hybridization, at first, the results of fragmented biotin-labeled cRNA probes were evaluated using a test chip (Test 2 Chip) to confirm that there was no problem. After that, this examination was performed. In this examination, rat chip sets (RG-U34A, RG-U34B, and RG-U34C) were used. In these three rat chip sets, there were 7000 kinds of known rat genes and 17000 kinds of unknown rat genes in total. Each of the test chip and rat chip set was subjected to hybridization in the following procedures.

60 μg of a fragmented biotin-labeled cRNA probe, 12 μl of control oligonucleotide B2 (5 nM), 12 μl of 100 x control cRNA cocktail, 12 μl of herring sperm DNA (10 mg/ml), 12 μl of acetylated BSA (50 mg/mg), and 600 μl

of a 2 x MES hybridization buffer were added and adjusted to 1200 μl in volume with the DEPC processing solution (hereinafter, referred to as a "hybridization cocktail"). The hybridization cocktail was thermally denatured by heating at 99°C for 5 minutes. After standing at 45°C for 5 minutes, the resultant was centrifuged at 15000 rpm at room temperature for 5 minutes. A supernatant was used for hybridization such that a 80-μl aliquot of the supernatant was sampled in the test chip (Test 2 Chip) and a 200-μl aliquot thereof was sampled in the rat chip sets (RG-U34A, RG-U34B, and RG-U34C).

The Gene chip was cooled to room temperatures, and then pre-hybridization was carried out with 1 x MES buffer (80 μl for Test 2 chip and 200 μl for rat chip set) at 60 rpm at 45°C for 10 minutes. Then, the pre-hybridization solution was removed and added with the thermally denatured hybridization cocktail, allowing the hybridization at 60 rpm at 45°C for 16 hours.

(3) Washing, dyeing, and scanning

The hybridization cocktail was removed from the Genechip and then a non-stringent wash buffer was added therein, followed by washing and dyeing with Fluidic station (manufactured by Affimetrix Co., Ltd.). The washing and dyeing were performed in the Test 2 Chip according to the Fluidic station Mini_euk1 protocol and in the Rat chip set according to the EukGE-WS2 protocol, respectively. After completing the washing and the dyeing, the chip was scanned by a scanner to take in image data.

(4) Data analysis

The data analysis on the hybridization was performed using the GeneChip analysis suite. The results are listed in Tables 1 to 4, respectively. The expression amount of each gene is represented by an average difference such that the average of the total gene expressions is defined as 100. In the table, the probe set number is denominated by Affymetrix Co., Ltd., which is the administrative number corresponding to each gene. The Unigene is an assembly in which DNA sequences registered in the GenBank are grouped into the category of gene (translation product) species and biological species.

Regarding the column in the table directly after the isolation, hepatic stellate cells were separated and seeded on a non-coating plastic petri-dish. After 4 hours, RNA was prepared from the hepatic stellate cells. The columns on the third day and seventh day correspond to the gene expression levels of cells at the initial stage of activation and activated stellate cells after incubated for 3 days and 7 days, respectively, in the non-coating plastic petri-dish.

There were 105 kinds of known genes having a significant increase in gene expression of the activated hepatic stellate cells incubated on a non-coating plastic petri-dish (increased about 3-fold or more) as compared with the expression level of the hepatic stellate cells in the resting state directly after isolating from the liver in the normal state.

Table 1

gene number	Probe set number	Unigene number	directly after isolation	3rd day	7th day	name of gene
1	L19927_at	Rn.9723	87	390	421	ATP synthase gamma-subunit (ATP5c)
2	U00926_g_at	Rn.3879	101	466	271	delta subunit of F1F0 ATPase
3	rc_AI008106_at	Rn.3233	231	1668	1559	calcium/calmodulin-dependent serine protein kinase
4	U17565_g_at	Rn.10220	21	189	125	intestinal DNA replication protein
5	rc_AA899854_at	Rn.5821	74	366	206	Topoisomerase (DNA) II alpha
6	rc_AI228738_s_at	Rn.2792	211	930	957	FK506-binding protein 1 (12kD)
7	rc_AI228045_at	Rn.11065	-4	996	1144	regulator of G-protein signaling 4
8	rc_AI229727_at	Rn.1150	83	410	405	regulator of G-protein signaling 5
9	rc_AA851814_at	Rn.13778	308	1767	1748	osteoactivin
10	S49003_s_at	Rn.2178	-41	243	293	short isoform growth hormone receptor [rats, mRNA, 1136 nt]
11	AF023621_at	Rn.11286	7	117	91	sortilin
12	M32062_at	Rn.6050	52	302	550	Fc-gamma receptor
13	J05122_at	Rn.1820	103	692	267	peripheral-type benzodiazepine receptor (PKBS)
14	AB017711_at	Rn.28212	18	202	121	RNA polymerase II
15	M36410_g_at	Rn.6658	74	701	284	sepiapterin reductase
16	AF041066_at	Rn.22804	15	111	133	ribonuclease 4
17	X06916_at	Rn.504	32	730	486	p9Ka homologous to calcium-binding protein
18	rc_AA819338_at	Rn.1999	115	536	624	sepiapterin reductase
19	M80829_at	Rn.9965	45	457	383	Troponin T, cardiac
20	rc_AI013887_at	Rn.2060	145	695	531	BCL2/adenovirus E1B 19 kDa-interacting protein 3 (Bnip3) ; nuclear gene for mitochondrial product
21	rc_AI009801_at	Rn.2661	77	346	156	macrophage migration inhibitory factor (Mif)
22	L02530_at	Rn.9095	14	147	178	polarity gene (frizzled) homologue
23	rc_AI170366_at	Rn.860	150	752	423	HEPATOMA-DERIVED growth factor
24	M69055_at	Rn.6431	23	167	149	insulin-like growth factor binding protein (rIGFBP-6)
25	X06107_i_at	Rn.6282	6	426	577	insulin-like growth factor I
26	rc_AI234060_s_at	Rn.11372	66	883	1025	Lysyl oxidase
27	M14656_at	Rn.8871	25	1039	988	osteopontin
28	H32867_at	Rn.9526	67	549	283	secretory leukocyte protease inhibitor (SLPI) mRNA, complete cds
29	AF014827_at	Rn.10796	37	160	264	vascular endothelial growth factor D (VEGF-D)
30	X58865mRNA_at	Rn.10981	83	433	264	liver phosphofructokinase

Table 2

gene number	Probe set number	Unigene number	directly after isolation	3rd day	7th day	name of gene
31	X02610_at	Rn.4236	279	914	798	non-neuronal enolase (NNE) (alpha-alpha enolase, 2-phospho-D-glycerate hydrolase EC 4.2.1.11)
32	rc_AI228723_s_at	Rn.1383	89	443	224	phosphoglycerate mutase B isozyme (PGAM)
33	rc_AA998722_s_at	Rn.1556	194	1298	770	Pyruvate kinase, muscle
34	D89514_at	Rn.11052	24	142	131	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
35	rc_AI233173_at	Rn.6236	50	297	154	nucleoside diphosphate kinase beta isoform
36	U64030_at	Rn.6102	-1	274	142	dUTPase
37	D00680_at	Rn.1491	195	712	567	plasma glutathione peroxidase
38	rc_AI170353_at	Rn.2554	234	1286	1188	p75NTR-associated cell death executor (Nade)
39	M60753_s_at	Rn.220	-2	440	318	Catecholamine-O-methyltransferase
40	rc_AA859911_g_at	Rn.23404	32	150	167	gal beta 1,3 galNAc alpha 2,3-sialyltransferase
41	rc_AI030409_f_at	Rn.54684	171	965	936	Calreticulin
42	X76985_at	Rn.11404	10	245	261	latexin
43	J03752_at	Rn.2580	19	149	151	glutathione S-transferase
44	rc_AI230260_s_at	Rn.11095	60	298	201	casein kinase II beta subunit (CK2)
45	rc_AA817897_s_at	Rn.2024	195	797	544	BCL2/adenovirus E1B 19 kDa-interacting protein 3 (Bnip3)
46	E12625cds_at		17	169	119	novel protein which is expressed with nerve injury.
47	D13127_g_at	Rn.1817	211	874	621	oligomycin sensitivity conferring protein
48	D32209_at	Rn.10123	24	170	96	Acid nuclear phosphoprotein 32 (leucine rich)
49	rc_AI112012_at	Rn.13778	542	1680	1987	osteoactivin
50	U75917_g_at	Rn.7160	56	415	383	clathrin-associated protein 17 (AP17)
51	U96130_at	Rn.3661	11	124	145	glycogenin
52	rc_AA899914_s_at	Rn.22161	-40	137	166	glycoprotein processing glucosidase I
53	M60322_g_at	Rn.2917	75	341	327	aldose reductase
54	M91597_s_at	Rn.927	368	1321	968	substrate binding subunit of type II 5'-deiodinase D2p29
55	AB016800_at	Rn.228	23	147	139	7-dehydrocholesterol reductase
56	D37920_at	Rn.11036	24	153	152	squalene epoxidase
57	M27207mRNA_s_at	Rn.2953	188	1092	1384	alpha-1 type I collagen
58	X05834_at	Rn.1604	89	806	1256	fibronectin
59	M83107_at	Rn.774	44	1033	849	signal sequence receptor, delta
60	M15474cds_s_at		84	611	979	alpha-tropomyosin

Table 3

gene number	Probe set number	Unigene number	directly after isolation	3rd day	7th day	name of gene
61	M60666_s_at	Rn.1033	103	562	762	alpha-tropomyosin 2
62	rc_AA944275_i_at	Rn.54749	122	556	262	alpha-tubulin
63	X73524_at	Rn.1657	40	236	33	desmin
64	rc_AA946377_at	Rn.1239	102	460	511	nonmuscle myosin heavy chain-B mRNA
65	rc_AI175789_at	Rn.6321	8	295	368	smooth muscle alpha-actin
66	X06801cds_i_at		6	1219	1301	vaskular alpha-actin
67	rc_AI009426_at	Rn.10	292	359	1338	SM22
68	S83358_s_at		27	170	205	focal adhesion kinase/pp125FAK/FAK
69	X60767mRNA_s_at	Rn.6934	14	168	66	cdc2
70	D14014_g_at	Rn.947	39	20	354	cyclin D1
71	D16309_at	Rn.9483	150	594	692	cyclin D3
72	L11007_at	Rn.6115	176	692	711	cyclin-dependent kinase 4 (cdk4)
73	X06564_at	Rn.11283	-46	231	428	140-kD NCAM polypeptide
74	X62660mRNA_at		11	147	144	glutathione transferase subunit 8
75	S69874_s_at		216	858	940	cutaneous fatty acid-binding protein
76	L03294_at	Rn.3834	71	336	722	lipoprotein lipase
77	S81497_s_at		42	241	247	lysosomal acid lipase
78	U67995_s_at/AF036761_at	Rn.2627	53	229	176	stearyl-CoA desaturase 2
79	rc_AI230712_at	Rn.950	-21	123	159	Subtilisin - like endoprotease
80	rc_AA850334_at	Rn.11187	14	100	70	Sulfonylurea receptor
81	AJ007291_g_at	Rn.30105	105	405	272	CAP1
82	rc_AI169631_s_at	Rn.29754	4	169	107	prohibitin (phb) mRNA
83	X98517_at	Rn.10516	56	531	1017	macrophage metalloelastase (MME)
84	D30804_g_at	Rn.19891	167	635	512	proteasome subunit RC6-1
85	D10952_i_at	Rn.6686	129	598	433	cytochrome c oxidase subunit Vb
86	M89945mRNA_at		124	570	357	farnesyl diphosphate synthase
87	rc_AI180442_at	Rn.2622	-2	152	81	testis-specific farnesyl pyrophosphate synthetase
88	rc_AA892775_at	Rn.2283	63	610	853	Lysozyme
89	D00753_at	Rn.128	10	981	443	contrapsin-like protease inhibitor
90	rc_AI227671_at	Rn.7219	27	194	215	steroidogenic acute regulatory protein (StAR) mRNA, complete cds

Table 4

gene number	Probe set number	Unigene number	directly after isolation	3rd day	7th day	name of gene
91	rc_AI228830_s_at	Rn.24366 /Rn.2627	127	826	496	Scd2 mRNA for stearoyl-CoA desaturase 2
92	rc_AA900582_at	Rn.780	158	197	929	Alpha-2-macroglobulin
93	S87522_g_at		-16	360	435	leukotriene A4 hydrolase
94	D14441_at	Rn.55102	99	383	360	NAP-22 mRNA for acidic membrane protein of rat brain
95	L16532_at	Rn.31762	29	222	157	2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPII)
96	L11319_at	Rn.24875	52	262	193	signal peptidase
97	D00569_g_at	Rn.2854	11	163	100	2,4-dienoyl-CoA reductase
98	D00729_g_at	Rn.48805	-36	157	110	delta3, delta2-enoyl-CoA isomerase
99	AA799336_at	Rn.1318	27	183	138	Moderately similar to Acyl carrier protein, Mitochondrial
100	D00636Poly_A_Site#1_s_at		153	570	508	NADH-cytochrome b5 reductase
101	AA685112_at	Rn.3373	15	149	123	similar to NADH-ubiquinone oxidoreductase
102	U62635_s_at	Rn.1608	-16	138	100	ribosomal protein L23-related product homolog
103	X59375mRNA_at	Rn.34330	211	861	936	ribosomal protein S27
104	rc_AA875269_at	Rn.2627	67	487	440	ribosomal protein L21
105	D30649mRNA_s_at	Rn.44	2	280	193	phosphodiesterase I

Example 2

With the method as described in <1> of Example 1, resting-state hepatic stellate cells were prepared and incubated for 24 hours in a MEM medium containing 5% FBS. Then, the medium was replaced with a fresh MEM medium containing 5% FBS or the same medium additionally containing 10 mM cysteine and the stellate cells were further incubated for 48 hours. The medium was removed from the petri-dish. After washing the petri-dish with PBS, cells adhered on the petri-dish were dissolved by Isogen and the RNA thereof was prepared by the method described in <2> of Example 1, followed by the Gene Chip measurement and data analysis by the method described in <3> of Example. The results of the analysis were described as the effects of cysteine to the activated initial hepatic stellate cells.

Similarly, resting-state hepatic stellate cells were prepared and incubated in a MEM medium containing 5% FBS. The incubation was conducted for 3 days, while the medium was replaced with a fresh MEM medium containing 5% FBS every day. After 3 days, it was replaced with a fresh medium that contains 5% FBS or replaced with the same medium additionally containing 10 mM cysteine, followed by incubation for 2 hours or 8 hours. The medium was removed from the petri-dish. After washing the petri-dish with PBS, cells adhered on the petri-dish were dissolved by Isogen and the RNA

thereof was prepared, followed by measuring and analyzing the resultant with Gene Chip. The results of the analysis were described as the effects of cysteine to the hepatic stellate cells at the middle stage of activation.

Similarly, resting-state hepatic stellate cells were prepared and incubated in a MEM medium containing 5% FBS. The incubation was conducted for 7 days, while after 3 days and 5 days, the medium was replaced with a fresh MEM medium containing 5% FBS. After 7 days, the medium was replaced with a fresh MEM medium containing 0.1% FBS. After 9 days, it was replaced with a fresh medium that contains 0.1% FBS and PDGF (human recombinant platelet-derived growth factor, manufactured by Sigma Co., Ltd.) at a final concentration of 20 mg/ml or replaced with the same medium additionally containing 10 mM cysteine, followed by incubation for 6 hours or 24 hours. The PDGF was added for the purpose of further accelerating the proliferation of hepatic stellate cells which were decreased in proliferation capacities due to the activation. The medium was removed from the petri-dish. After washing the dish with PBS, cells adhered on the petri-dish were dissolved by Isogen and the RNA thereof was prepared, followed by measuring and analyzing the resultant with Gene Chip. The results of the analysis were described as the effects of cysteine to the hepatic stellate cells at the later stage of

activation.

In each of the initial, middle, and later stages of the activation of hepatic stellate cells, the influences of cysteine on the gene expression are shown in Tables 5 to 7. In Tables 5 to 7, the gene numbers are similar to those shown in Tables 1 to 4. Among the genes whose expression levels are increased as the hepatic stellate cells are activated as shown in Tables 1 to 4 in Example 1, the genes where the inhibiting effects of cysteine are observed at each of the initial, middle, and later stages of the activation are shown in Tables 5 to 7. When the expression inhibiting effects are found in the genes at the initial, middle, and later stages of the activation, then the respective genes are evaluated as and the expression intensities (average differences) are illustrated.

Cells incubated for 24 hours after the preparation of hepatic stellate cells were used as hepatic stellate cells at the initial stage of activation. In addition, the measurement value of the cells was described as "initial/initial (24 hr)"; the measurement value of the cells incubated for 48 hours while the medium was replaced with a fresh medium was described as "only medium/initial (+48 hr)"; and the measurement value of cells incubated for 48 hours in the medium added with cysteine was described as "cysteine added/initial (+48 hr)", respectively.

Furthermore, cells incubated for 3 days after the preparation of hepatic stellate cells were used as hepatic stellate cells at the middle stage of activation. In addition, the measurement value of the cells was described as "initial/middle (day 3)"; the measurement value of the cells incubated for 2 hours or 8 hours while the medium was replaced with a fresh medium was described as "only medium/middle (+2 hr)" or "only medium/middle (+8 hr)"; and the measurement value of cells incubated for 2 hours or 8 hours in the medium added with cysteine was described as "cysteine added/middle (+2 hr)" and "cysteine added/middle (+8 hr), respectively.

Furthermore, cells incubated for 9 days after the preparation of hepatic stellate cells were used as hepatic stellate cells at the later stage of activation. In addition, the measurement value of the cells was described as "initial/later (day 9)"; the measurement value of the cells incubated for 6 hours or 24 hours while the medium was replaced with a fresh medium was described as "only medium/later (+6 hr)" or "only medium/later (+24 hr)"; and the measurement value of cells incubated for 6 hours or 24 hours in the medium added with cysteine was described as "cysteine added/later (+6 hr)" and "cysteine added/later (+24 hr), respectively.

As shown in Tables 5 to 7, the cysteine exerted an

influence on the expressions of many genes. The cysteine gave effects on the process of activating hepatic stellate cells. Thus, it is predicted that cysteine inhibits hepatic fibrosis. The fact that the cysteine actually inhibits hepatic fibrosis will be described below.

Hepatic fibrosis was induced by intraperitoneally administering 10 mg/kg of dimethylnitrosamine (DMN) to a 6-week SD male rat three times a week for four weeks. Then, an experimental diet containing 0.5% of L-cysteine (Cys) as a subject and that containing 0.5% of casein as a control were fed from the initiation date of the DMN administration. On the 28th day from the initiation of the DNA administration, the liver was sampled from the subject and the amount of hydroxyproline (Hyp) in the liver was measured as an index of hepatic fibrosis using an amino acid analyzer. The results are shown in Fig. 1. As was evident from the figure, an increase in Hyp in the liver, which was increased 6-fold by the administration of DMN, was significantly suppressed by the oral administration of Cys.

As described above, the gene panel of the present invention allows cysteine to inhibit the hepatic fibrosis, where the cysteine has medicinal benefits to change genetic variations required in the process of activating hepatic stellate cells. From the above, it is confirmed that a screening method using this gene

panel is an effective screening method.

Table 5

gene number	effect in initial stage	effect in middle stage	effect in later stage	initial initial (24hr)	only medium initial (+48hr) control	cysteine added initial (+48hr) +Cys	initial middle (day3)	only medium middle (+2hr) control	cysteine added middle (+2hr)	only medium middle (+8hr)	cysteine added middle (+8hr)	initial later (day9)	only medium later (+6hr)	cysteine added later (+6hr)	only medium later (+24hr)	cysteine added later (+24hr)
1	○	○	○	283.4	411.2	374.4	353.7	469	391.4	421.4	321.9	338.5	347.9	230.2	402.1	314.8
2	○	○	○	439.3	519.6	355	515.4	510	345.9	680.6	317.4	338.9	763.6	231.7	544.9	387.6
4	○	○	○	82.3	167.5	84.6	122.9	135.3	98.4	130.3	104.1	54.7	62.7	17.9	54.4	20.3
5	○		○	33.3	89.2	2.2	26.9	26.8	30.3	32.3	27.7	45.3	54.2	14.7	47.4	38.6
6		○		975.3	978.7	844.2	1533.5	1242.3	1400.6	1948	1310.8	714	135.6	857.8	929.2	1015.9
7			○	421.7	183.6	1.7	7	1.8	0.8	3.2	0.9	72.7	588.6	10.8	71.4	16.3
10			○	41.8	43.7	3.2	32.2	39.2	17	5	6	88.9	101.9	8.6	56	57.2
11	○	○		18.3	42.1	6.4	7.4	11.8	14.3	15.7	6.4	58.8	12.9	12.7	27.6	39.7
13	○		○	188.1	661.6	363.4	541.8	439.2	351.3	380.9	342.4	222.6	802.8	406.7	408.4	370.6
15	○	○	○	76	178.4	85.3	92.1	50.9	29.3	107.2	61.7	92.3	232	43.7	155.9	64.4
16	○			76.2	91.5	40	34.5	47.1	59.2	37.3	38.9	176.8	292.1	399.7	155	212.5
17	○	○	○	40.8	896.2	171.4	118.8	204.5	179.1	246.1	178.9	152.9	729	387.7	547.6	530
18	○			237.1	436.8	306.6	314	355.4	425.5	340.7	298.6	588.3	564.3	470.4	469	465.9
19	○		○	13	212.3	9.8	12.5	13.8	15.8	16.6	19.7	304.2	892.2	66.4	857	745.7
21				231.8	279.3	491	335.8	252.3	316.9	309.8	346.1	139.2	319.6	287	234.2	234.3
22	○	○		22.3	99.2	13.7	32.7	12.2	7.6	43.7	8.8	159.5	44.4	18.8	21.8	17
24	○		○	35.5	58.5	21.6	31.5	36.7	29.9	24.7	40.8	45.7	96.7	85.9	90.2	74.6
25	○			6.7	43.5	24.8	8.4	6.5	10.9	4.6	8.4	130.4	21.5	5	75.8	68.1
26	○	○		197.4	864.7	15.8	269.3	283.1	151.1	304	87.5	2129.5	1710.1	251.9	1498.5	1075.7
27	○		○	18.3	2002.1	1337.4	128.3	216.1	262.3	167.7	179.5	10.7	431.1	238.8	709.9	416.4
29	○	○		38.3	103	13.5	4.5	4.4	17	33.4	24.6	60.5	54.8	11	156.7	145.9
30			○	215.4	233.8	364.1	137.3	93.1	138	168.2	158.8	112.5	156.4	103.2	74.6	66.5
35	○		○	194	382.5	295.5	369.4	464.5	457.9	544.7	591.3	281.3	467.5	201.8	281.1	285.5
37	○	○		42.1	449.7	1.9	37.4	49.5	63.7	90.6	42.1	388	320.2	22.3	145.2	147
40	○	○		67.9	97.2	22.3	88.9	81.9	13.1	91.4	36	168.4	73.2	8.4	88.9	32.1
42	○			18	173.6	79.4	43.5	37.1	65.5	58	52.4	430.8	205.2	152	181.5	239.7
46	○	○	○	321.8	414	295.4	351.8	660.2	392.2	301.9	270.2	1005.6	1388.3	569.8	871.1	755.3
47		○	○	577.4	888.5	907.4	1373.6	1106	1072.3	1557.4	1002	603.4	490.2	380.2	1423.3	1053
50			○	299	286.4	221.1	206.3	160.4	124.5	152.4	100.1	267.7	438.7	126.5	234.8	144.2

Table 6

gene	effect in	effect in	effect in	initial	only medium	cysteine added	initial	only medium	cysteine added	only medium	cysteine added	initial	only medium	cysteine added	only medium	cysteine added
number	initial stage	middle stage	later stage	initial (24hr)	initial (+48hr) control	initial (+48hr) +Cys	middle (day3)	middle (+2hr) control	middle (+2hr)	middle (+8hr)	middle (+8hr)	later (day9)	later (+6hr)	later (+6hr)	later (+24hr)	later (+24hr)
53		○		441.1	723.8	1097.8	672.7	651.1	885.9	909.1	608.6	496.8	218.1	1562.5	236.5	460.8
55	○	○	○	41	89	26.7	31	26.6	28.7	49.7	17.4	80.5	150.1	36	77.4	64.1
57	○		○	197.3	1676.8	24.6	579.8	570.9	416.1	513.6	436.6	2062.8	2151.9	1018.8	2191.4	1457.1
58	○		○	1077.7	2164.1	386.5	1151.4	1383.8	1357.3	1254.5	1540.1	3061.3	4315.7	3550.1	4520.7	3750.6
59	○	○	○	1345.1	3236.2	377.5	2132.6	2435.8	2274.1	3103.7	2548	3265.3	3327.1	989.1	3334.3	2442.7
60	○			497.4	871.5	63	562.4	501.1	385.8	386.1	683.8	1721.5	330.1	475.7	1190.9	1254.2
61	○		○	618.4	1091.7	74.9	873.9	669.8	692.3	614.8	972.9	1811.7	1224.1	586.8	1961.5	1869.8
62			○	1297.2	1975.4	1940.6	2469.2	2553.7	3104.4	3918.1	3953.3	582.9	2365.4	1840.2	2760	1917.5
63	○	○	○	86.8	554	120.1	233.2	428.2	355.1	500.3	464.4	9.3	254.6	179	147.6	134.2
65			○	2188.1	2173.2	205.6	1769	1868.1	1617.8	1908.3	2763.4	2381.4	1414.5	678.4	2917.4	1631.3
66	○	○		2775.2	3798.4	53.7	1876.3	1172.2	1945.9	3455.4	2623.3	3864.1	1117.8	531.8	2165.2	2529.5
68	○	○	○	103.2	212.1	113.2	224.4	211.2	135.1	293.8	169.5	337.6	454.2	99.2	518.9	186.7
69	○			20.1	43.1	3.6	16.9	15.4	16.7	16.7	10.7	35	12.5	13.3	35.5	30.7
70	○	○	○	11.6	206.7	22	59.2	68.4	30.2	175.7	25.4	403.2	283.3	79.2	650.3	543.8
71	○	○		94.2	477.2	74.9	156	121.3	128.2	429.2	123	413.3	246.5	55.3	213.7	134.2
72	○			612.6	427	324.7	445.6	465.8	292.1	429.3	280.6	479.8	406.8	434.5	446.7	425.5
73	○			15.4	87.4	6.4	40.2	18.5	28.3	34.2	32.4	257.3	145.5	10.9	257.4	198.3
74		○		305.1	27.8	120.5	68.8	79.2	32.8	31.8	38.3	3	3.6	3.3	3.1	11.5
76	○	○		88.6	249.8	136.6	192.3	234.5	218.6	210.2	163.1	402.6	313.6	93	307	339.9
79	○			76.5	77.4	2.5	44.5	50.9	33.8	30.4	33.2	261.4	95.3	42.1	189.7	176.7
81			○	477.1	392.3	456.8	535.9	443.7	391.8	401.1	385.4	290.3	388.8	223.6	332.2	380.8
82			○	300.9	182.1	506.8	368.1	364.6	388.1	337.7	289.7	190.8	475.5	260.8	212.6	239.8
83		○	○	62.1	208.9	390.9	60.3	101.8	74.3	52.9	41.5	29.2	616.9	365.4	484.2	496.3
84		○	○	630.9	556.1	864.5	818	850.2	568.6	752	594.9	398.5	915.4	442.1	610.6	576.2
85			○	300.2	316.8	234.7	264.2	239.9	297.5	278.2	229	247.4	226.7	102.9	185.7	124.8
86	○		○	308.2	527.2	343.5	372.5	426.3	374.5	307.5	449	921.9	2310.9	663.5	1088.7	1499.9
87	○			47.2	71.8	36.1	45.5	56.3	84.5	57.3	77.4	249.8	24.5	131.9	187.4	183.1
88			○	16.7	513	763.8	51.1	101.1	100.4	73.6	69.7	113.8	2666.2	1555	3019.8	3143.1
89		○	○	2893.4	256.3	143	1988.2	2019.3	1009.6	1273.9	1364.3	172.7	215.9	109.6	88.8	96

Table 7

gene	effect in	effect in	effect in	initial	only medium	cysteine added	initial	only medium	cysteine added	only medium	cysteine added	initial	only medium	cysteine added	only medium	cysteine added
number	initial stage	middle stage	later stage	initial (24hr)	initial (+48hr) control	initial (+48hr) +Cys	middle (day3)	middle (+2hr) control	middle (+2hr)	middle (+8hr)	middle (+8hr)	later (day9)	later (+6hr)	later (+6hr)	later (+24hr)	later (+24hr)
92			○	55.2	51.7	32.2	43.9	38.6	34.9	40.4	36.8	94.9	510.6	71.6	194.4	214.8
93		○	○	489	665.1	834.7	510.2	577.4	303	586.7	382.3	295.8	891.5	187.2	725	564.4
94	○		○	236.5	546.7	170.7	786.2	674	573.2	733.7	658.9	1031.4	1006.3	826.2	1141.7	1211.8
95	○			135	106.7	19.5	56.4	34.3	20.6	57.4	40	76.5	27.3	19.8	37.5	26.4
96		○		265.2	266.7	314.4	163.4	237.3	189.2	133.3	181	283.8	309.4	292.1	233.8	258.5
97			○	113.4	159.8	109.2	123.1	67.6	84.5	86.9	117.1	107.5	37.7	26	117.6	64.8
98			○	223.9	151.4	89.2	173	71.7	115	116.1	130	113.2	96.8	36.5	123.8	101.3
99	○			98.8	103.5	78.6	110.8	124.6	67.2	81.7	37.3	107.5	88.5	28.8	89.5	47.2
100	○			306.7	408.8	148.6	324.9	262.7	235.4	305.2	308.3	455.7	5.4	291.4	301.9	418.4
102			○	266.5	248.5	148.2	374.9	348	185	246.9	201.2	212.1	231	69.6	213.7	129.7
103		○		899.8	1288.2	1731	1378	1413.7	1361.6	1606.9	1277.1	1285.7	1794.4	1765.2	898.1	1268.3
104	○		○	132.1	163.7	58.6	65.3	43.6	54	34.1	46.5	607	943	118.2	520.5	301.5

Industrial Applicability

According to the present invention, there is provided the information about the expression of a gene related to hepatic stellate cells. Using the expression information, the screening of drugs or the like to obtain one capable of inhibiting the activation of the hepatic stellate cells can be carried out.